THE BEHAVIOR OF CELLS IN TISSUE CULTURES OF FUNDULUS HETEROCLITUS WITH SPECIAL REFERENCE TO THE ECTODERM.[1]

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INTRODUCTION.

Human, mammalian, avian and amphibian tissues have been frequently employed for the study of cells in tissue cultures but comparatively few observers (Osowski 1914, Lewis 1916 and Dobrowolsky 1916) have used fish tissue. These embryos are easily obtained, the culture media are simple to prepare, and large growth takes place at room temperature. They therefore constitute an ideal material in which to study the movement of the epithelial membrane, the structure of the ectoderm cells and their relation to other cells.

TECHNIQUE.

Pieces of *Fundulus* embryos were explanted into diluted sea water in the manner described by M. R. Lewis ('16). As an additional precaution against infection, the eggs were dropped into 95 per cent. alcohol for one second and then transferred to sterile sea water. In preparing the media several dilutions of sea water were employed: *i.e.*, 20 per cent., 30 per cent., 35 per cent., 40 per cent. and 50 per cent. in distilled water. To 80 c.c. of each of these dilutions was added 20 c.c. *Fundulus* bouillon, 0.02 gm. $NaHCO_3$, and 0.5 gm. dextrose. The media were then sterilized.

Growth was obtained in dilutions of sea water ranging from 20 to 50 per cent., although in the latter it was infrequent and not of great extent. While growth was satisfactory in a dilution of 20 per cent., the proportion of good growths was larger with 30 per cent. and still larger with 40 per cent. sea water. More-

[1] From the Marine Biological Laboratory, Woods Hole, Mass.

over, cell proliferation from each piece was more abundant. Cultures grew in media containing no dextrose, or with varying amounts of dextrose up to 2 per cent. Media containing 0.5 per cent. gave the best results. The cultures grew equally well with 80 per cent. of Locke's solution in place of sea water. Good results were obtained with 80 per cent. of Locke's solution and 20 per cent. of chicken bouillon substituted for the fish bouillon, although not so many cultures grew. Pure egg albumen and dilutions of this added to the normal medium likewise proved successful.

Several cultures which contained tissue from both chick and *Fundulus* embryos were made in Locke's solution, chicken bouillon and dextrose. These were kept in the incubator at 39° C. and at the end of 48 hours the explants showed equally good outgrowths.

Cultures were usually prepared in the afternoon, and by the following morning growths in the form of membranes had formed, though sometimes this did not occur until the second day, especially when a dilution of 20 per cent. sea water was used. The embryos were 7, 10, 14 and 15 days old (just before hatching) but the age made no apparent difference in the growth from the explant.

General Characteristics of the Cultures.

Examination of the cultures shortly after explantation usually showed a few isolated mesenchyme cells, which soon began to migrate outward on the cover-slip. The ectoderm cells in the region of the cut edges rounded up into more or less spherical bodies. After several hours typical cultures revealed a membranous outgrowth of ectoderm from one or more regions of the explant, and beyond this an area of mesenchyme cells, isolated, or forming a reticulum upon the under surface of the cover-glass (Fig. 1). Nerve fibers, projecting freely into the fluid along the coverglass, were often present in abundance. Pigment cells and yolk-filled cells from the digestive tract (Fig. 7) were also common. No outgrowth of muscle cells was observed.

A renewal of the fluid medium was not attempted in any of the cultures. The oldest healthy cultures were ten days old,

and in one of these the heart was still beating at the time of fixation. In one culture peristalsis was observed in a portion of the intestine which projected out into the medium. Contractions of the trunk musculature, beating of the heart, and movements of the fins were frequently observed in cultures several days old.

Behavior of Ectoderm Cells at Cut Edges.

In normal embryos the ectoderm consists of a single layer of large pavement cells, polygonal in surface view, which may be clearly seen in the regions covering the fins and the trunk musculature. The cells are transparent and almost colorless. The internal structures are only vaguely visible. The cells covering the trunk have delicate concentric markings, suggesting somewhat the markings upon fish scales.

When the embryos are cut the ectoderm cells along the edge round up very markedly into spherical masses, especially in the region of the heart and the yolk sac. Any small group of cells or single cells that have in cutting become separated from the explant remain for some time in this state. Eventually, large vacuoles develop in them and they remain inactive in this condition for several days before disintegration takes place. Similar cells were frequently observed in old cultures along the edge of the ectodermal membrane.

The direction of the cut is a factor in the successful growth of cultures from the trunk region of the body. The cells do not grow out unless the cut is irregular or oblique. If it is transverse the cells close in and form a covering over the injured end, preventing outward migration. Osowski found that injured surfaces of fish embryos were covered by an epithelial membrane within twenty-four hours.

Membranes never grow out from cut fin surfaces; the cells near the cut surface become wrinkled and irregular in contour, and remain in this condition sometimes for several days, before they become rounded up and display the large vacuoles and greenish protoplasm which are characteristic of degenerating ectoderm cells. Sometimes the ectoderm of the fins degenerates

even when not cut, although the other cells of the culture exhibit normal activity.

Ectodermal Membrane.—Fig. 1 shows the extent of a characteristic membrane in a seven-day culture. In the living cultures the ectodermal cells spread out in a very thin, flat and colorless layer on the under side of the cover-glass. The position of the nucleus and of the granules within the cell could rarely be detected. At the edge of the membrane the cells were thicker and darker in appearance, with a greenish tinge and of very irregular form. Projecting beyond the ectoderm, mesenchyme cells could be seen adhering closely to the cover-glass. Other cells, slightly darker and containing vacuoles and granules, were observed migrating above the ectodermal layer. Slides stained with iron hæmatoxylin show that such outgrowths from the explant consist of a practically continuous ectodermal membrane of extremely flat, slightly granular cells, and a more or less imperfect membrane of mesenchyme lying above it, closely adhering to the cover-glass (Fig. 2). The mesenchymal membrane is never so perfect as the ectodermal layer, and there is a gradual transition from a membranous form to more or less isolated cells which may project for a considerable distance beyond the ectoderm (Fig. 3). One group of ectodermal cells was found which was not covered on its upper surface by mesenchyme (Fig. 8). These cells were characterized by very granular nuclei containing one or more nucleoli, and by mitochondria in the form of threads and granules. Frequently the cells showed a rosette-like arrangement around a small intercellular space. Mesenchyme cells were readily distinguishable in the stained slides by their more granular cytoplasm and indefinite cell boundaries. The size of the nuclei was not a criterion, as the nuclei might be either larger or smaller than those of the ectoderm. Mitochondria were much more abundant than in the ectoderm and stained more deeply. Fig. 9 shows a group of mesenchyme cells, unaccompanied by ectoderm, in which the mitochondria appear very clearly. They were also observed in the living cells when stained with janus green. Certain other granules became visible when stained with neutral red. The latter stain also affected granules in the thickened

cells at the edge of the ectoderm, but the presence of such granules in the flat ectodermal cells could not be determined with certainty. These cells appeared less easily penetrable to a number of vital dyes, while the mesenchyme cells were readily colored.

Formation of Ectodermal Membrane.—In a study of fetal skin growing in blood serum, Loeb ('12) observed that the ectoderm cells migrated into the surrounding medium in the form of strands. Holmes ('13) and Uhlenhuth ('14) described a similar condition in the frog. In my observations on fundulus the ectodermal outgrowths were invariably in the form of very thin, one-layered membranes. The ectoderm never migrated in strands. The earliest appearance of the membrane was indicated by an exceedingly thin, flat layer near the explant which was continuous with the rounded cells covering the body region, and bordered along its outer edge by a broad mass of irregular thickened cells. The mesenchyme cells lay in a thin sheet above the ectoderm and projected beyond it. As the rounded cells migrated from the explant their contour changed gradually, and they became flattened with irregular, thickened, central portions which projected downward in the fluid medium. Text-figure 1 is a drawing of an ectodermal cell in the process of flattening out during its migration. The portion of the cytoplasm spread out along the cover-glass formed a clear thin area bounded by a cell wall which was in close contact with the walls of neighboring cells. In this clear region were a few pale concentric markings. The central mass of thickened cytoplasm eventually disappeared as the cell flattened out completely. A few hours later, as a result of the migration and flattening of the cells, the membrane had increased more than twice in extent, and no thickened cells remained except a few scattered ones and a single row of elongated cells around the edge.

Changes at the Edge of the Ectodermal Membrane.—The edge of the ectodermal membrane was seen to undergo slow and continual changes during the active growth of the culture. Usually the cells were thickened and elongated or extremely irregular in form, with numerous blunt knobs projecting downward. Their cytoplasm appeared granular in contrast to the

apparently homogeneous protoplasm of the flat cells. They were observed to flatten out and thus extend the membrane. It seemed as though the cells were under unequal tension for as the membrane grew wrinkles formed in cells that had formerly been flat. The wrinkles could be differentiated from the cell thickenings previously described, for they were obviously folds in the membrane and often involved more than one cell.

Ectodermal pseudopodia, formed from a hyaline outer region of the cells, have been observed by Harrison ('10) and by Holmes ('13) in tissue cultures of the frog. In the ectoderm of fundulus, however, the entire cell is equally hyaline when stretched out flat, and the formation of pseudopodia was never observed.

The ectodermal membrane is not only very extensible, but elastic as well. In staining the cultures with vital dyes the greatest care was necessary to prevent its retraction, which often followed within a few seconds. This is also likely to occur if the slides are jarred. In one culture the ectoderm cells along the edge contracted and thickened, pulling along with them large portions of the mesenchyme membrane. This double membrane then rolled in upon itself and, as it tore loose from the cover-glass, could be seen adhering by short projections from certain of the cells. These in turn loosened and the membrane pulled in farther (Fig. 5). In such cases of mechanical disturbance of the culture the membrane later became reduced by the contraction of the ectoderm to a compact mass of cells which eventually disintegrated. Ruth ('11) describes the contraction of the edges of the growing epithelial cells during the healing of a wound in the skin of a frog in vitro.

Striations in Ectoderm Cells.—The most peculiar characteristic of the ectoderm cells is the presence of numerous delicate striations, more or less concentrically arranged, which form an intricate pattern over all the cell (Figs. 3. and 10). The markings did not appear on all of the cells and only occasionally were they sufficiently clear in the living cultures to be drawn with the camera lucida. When the cells began to spread out and migrate from the cut surface of the embryo, a few markings could be seen in the flat clear portion of the cell (text-fig. 1) and, as this region

increased in extent, more striations became visible. The markings appeared to be on the under surface of the cells, that is, farthest from the mesenchyme. The striæ were very definite, appearing as longer or shorter dark lines over the cell, varying only slightly in width, and never crossing a cell boundary. The wall between adjacent cells was distinctly double, so that each cell had its own complete investment. This was also clearly seen in a few cases where ectoderm cells had become separated from each other. Frequently one or more striæ near the periphery of a cell were situated parallel with the cell wall, while the inner ones were more irregular or arranged concentrically with reference to several points in the cell.

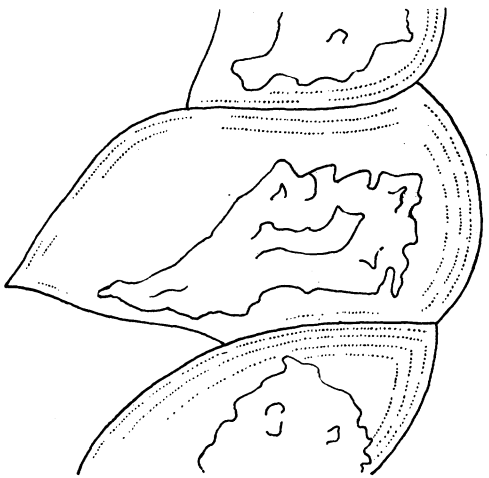

FIG. 1. Camera lucida drawing of an ectodermal cell in process of flattening out during migration; the central mass of thickened cytoplasm bordered by a clear flat region with pale concentric markings. Ocular 5, lens 4 mm.

Form and Behavior of Mesenchyme Cells.—The mesenchyme cells grew out from the explant as more or less elongated and separate cells, which later became connected in various ways. Adjacent cells sometimes sent out short broad processes along their sides, which appeared to fuse with each other, forming a reticulum with relatively small intercellular spaces (Fig. 9). Characteristic also of these cells was the projection of their protoplasm into short thread-like processes often giving a prickly appearance to the edge. The prickly processes became converted

into protoplasmic bridges connecting the cells. When these were present the tissue appeared somewhat similar to the mesothelial membrane described by Lewis and Lewis ('12) in tissue cultures of the chick, except that in fundulus the cell boundaries are much less distinct. Whether the cells actually fuse or not is a question which is extremely difficult to determine with certainty. When the cells are connected with each other by extremely elongated protoplasmic processes, as shown in Fig. 3, the intercellular spaces are large and a wide-meshed reticulum results. There is a gradual transition from a membranous to a reticular arrangement of cells as the distance from the explant increases.

The outward growth of these reticular cells appears to be an important factor in the extension of the ectoderm. As previously noted, reticular mesenchyme cells were usually seen projecting beyond the ectodermal membrane (Figs. 1 and 3). These cells underwent very marked changes in form, their protoplasm flowing in a direction away from the ectoderm, the proximal portions of the cells, however, being connected directly or by intervening cells with the thickened edge of the ectoderm. The distal portions of the cells frequently developed broad fan-like expansions, which were firmly anchored to the cover glass.

The mode of formation of the fan-like expansions appears to be about as follows: The pseudopodia flow out from the cell in the form of delicate, radiating, thread-like or finger-like processes; then the region between the processes gradually fills out until a fan-like form is attained. This soon becomes entirely homogeneous near the periphery and adheres closely to the cover glass. Frequently the fan has a slightly fluted appearance as if not adhering equally well at all points.

As the mesenchyme cells migrate outward they exert a pull upon the edge of the ectoderm which is drawn outward, not by amœboid processes of its own, but by amœboid processes and fan-like expansions of the mesenchyme cells to which it is attached. The sheet of ectoderm is thus anchored in all directions as if by minute guy-ropes. When these are pulled more in one direction, folds and wrinkles are formed, which become smoothed out when the pull is equalized.

Pigment Cells.—Chromatophores of fundulus are large cells containing either black, brownish red, or yellow pigment granules. The cells migrate readily from the explant, but do not as a rule travel far. Stockard ('15) showed that the chromatophores have an affinity for plasma-filled spaces, being found adhering to the pericardium in normal embryos and to the heart itself in embryos deprived of a circulation. In the cultures of fundulus brown chromatophores were several times observed closely adhering to the beating heart, which happened to be projecting out into the fluid medium. Here they remained elongated for several days until the cultures degenerated. Isolated pigment cells that had wandered out upon the membrane degenerated more rapidly than other kinds of cells, and the pigment granules, freed from the cells, were readily taken up by the mesenchymal cells where they became aggregated around the nucleus (Fig. 6).

In addition to the pigment cells and those forming a membrane or reticulum other types of mesenchyme cells were observed. Among these were certain denser, more granular and vacuolated cells which migrated out upon the mesenchymal reticulum, and other cells (probably clasmatocytes) with brighter, more solid-looking protoplasm and numerous curved, finger-like pseudopodia. These cells contained numerous granules often derived from degenerated chromatophores. Brownian movement of these ingested granules was frequently observed.

Yolk Cells.—In some explants certain peculiar spherical cells filled with numerous clear greenish yolk spheres were seen massed together in the anterior part of the digestive cavity. These cells migrated readily out upon the mesenchymal reticulum where they became very slowly amœboid and wandered out along the edge of the ectoderm. Fig. 7 shows a group of these cells with the yolk spheres stained deep black with iron hæmatoxylin.

Cell Division.—The appearance of new cell boundaries in the ectoderm was frequently observed but in no case was a cell seen to divide. There is therefore no evidence as to whether the cells divide by mitosis or amitosis. In over fifty cultures stained with iron hæmatoxylin no stages of mitosis were observed. Two

nuclei, however, were common in ectoderm cells (Fig. 8), as were also nuclei partly constricted into two, or with several irregular constrictions and variable nucleoli. There is no reason to believe that this condition is followed by division of the cells. It may be that the nuclei fuse together again, as Macklin ('16) observed in tissue cultures of the chick, and that the cells subsequently divide by mitosis. Holmes ('13) observed amitosis of the nuclei in tissue cultures of various tadpoles, but he states that nuclear division was not followed by division of the cytoplasm.

A few mesenchyme cells were observed to divide by mitosis and several groups of chromosomes in metaphase appeared in the stained material. Bi-lobed and double nuclei, indicative of amitosis, were also observed (Fig. 9).

Cultures of Chick and Fundulus.—A piece of fundulus tissue and a piece of muscle tissue from a chick embryo of eight days' incubation were placed together in a drop of Locke's solution containing chicken bouillon, and kept at 39° C. At the end of 48 hours each piece showed its characteristic form of growth, the fundulus having the double membrane previously described, the chick tissue showing the usual radiating type of outgrowth. In one region the fundulus outgrowth could be seen growing over a portion of the chick explant as over a foreign body. In another region outgrowths from the two pieces were almost in contact, but the cells from the two explants showed no tendency to intermingle. Specific differences were observable within the cells. Mitochondria are much more abundant in the chick tissues and the cytoplasm appears to be different, as shown by the greater ease with which the cellular structures of the chick may be observed.

General Considerations on the Movements of Membrane Cells.—The behavior of the cells in the cultures at different times is of considerable interest. Taking the normal form of the ectoderm cells as a standard, we find that the cohesive property of these cells is increased suddenly at the time the cut is made, as shown by the rounding up of the cells. The stimulus of the injury was sometimes effective for several hours. This influence seemed to

be gradually weakened and the adhesive power progressively increased as the cells spread out in a thin layer upon the under surface of the mesenchyme. Holmes ('13) found that ectoderm cells of tadpoles in tissue cultures attach themselves readily to various kinds of substrata, including the cover glass, and "extend upon one another in mutual attraction which tends to keep them in continuous masses." In fundulus, however, this stereotropic activity of the ectoderm is called forth only when the cells are associated with the mesenchyme, and it appears to be much stronger than in the frog, causing the cells to be spread out in a thin single-layered membrane. In over fifty cultures it was never observed that an ectodermal membrane grew out unaccompanied by mesenchyme, whereas numerous cultures contained growths consisting of mesenchyme alone. The latter cells appear to be more highly stereotropic than the ectoderm, for they will adhere to the smooth surface of the cover glass even to the extent of having their processes snapped off when the ectoderm retracts.

The difference in behavior of the two layers of cells is perhaps correlated with the fact that under normal conditions of development ectoderm cells grow only in contact with the mesenchyme, whereas mesenchyme cells can grow in contact with widely varying kinds of surfaces.

The question arises whether there is any relation between wound-healing and the formation of the ectodermal membrane in tissue cultures. Loeb ('20) has discussed various processes involved in cell movements in wound-healing, designating among others amœboid migration of ectoderm cells, this being "the first response of the tissue to the wound stimulus." A factor in this amœboid wandering of the ectoderm cells is their stereotropic reaction, as expressed by their contact with the coagulum, which is "the foundation for the process of wound healing." In fundulus cultures the wandering of the cells in contact not with a coagulum but in this case with the mesenchyme layer is the foundation for the process of formation of the ectodermal membrane. The manner of cell movement, however, does not appear to be amœboid in character.

Oppel ('13) has described the bending of the skin edges along the cut surface of explanted pieces of the tadpole's tail as due to real movement; not simply a mechanical process but a change in form by which the ectoderm grows around the cut. In an earlier paper ('12*a*) he compares the ectoderm cells to partly filled sacs of inelastic material which can change their outline without varying the extent of their surface. This kind of movement is strikingly similar to the early changes which take place when the rounded cells begin to flatten. Oppel ('12*b*) distinguishes between epithelial movement, which is a mass movement, and amœboid motion which tends to isolate cells, as in connective tissue. He concludes that while the movement of the epithelium depends on the activities of the cells themselves, it is not an amœboid motion. The observations on fundulus confirm this interpretation. Here epithelial movement appears to be a mass movement throughout all stages in the formation of the ectodermal membrane. Single functional epithelial cells are never found.

In conclusion it may be stated that the activities of the cells in the formation of the ectodermal membrane in fundulus are similar to activities displayed also by ectoderm cells in the process of wound-healing. As in the latter, the cells exhibit mass movements the end result of which is to cover the connective tissue; so in tissue cultures of fundulus the migration of the ectoderm cells proceeds by mass movement which results in a partial covering of the mesenchyme layer. The contact reaction toward underlying connective tissue, exhibited by ectoderm cells in wound-healing, is paralleled by the stereotropic activity of the ectoderm cells evoked by contact with the mesenchyme. It may be said that the cells are attempting to follow out their normal activities, although subjected to abnormal conditions.

Summary.

1. Tissues of *Fundulus heteroclitus* grew in fluid media under conditions varying widely in respect to temperature, concentration of salts, and character of nutritive substances.

2. Mesenchyme cells migrated out into the medium upon the under surface of the cover-glass and formed almost continuous

or reticular membranes with isolated cells lying beyond. The ectoderm formed a membrane in close contact with the under surface of the mesenchyme. Nerve fibers, pigment cells, and yolk cells from the digestive tract readily migrated out. Peristalsis of the intestine, beating of the heart, contraction of the trunk musculature, and movements of the fins, were observed in numerous cultures several days old.

3. Characteristic of the ectoderm cells were certain delicate striations somewhat concentrically arranged, which formed an intricate pattern over the cell.

4. The mesenchyme cells are highly amœboid and possess characteristic fan-like expansions by means of which they adhere to the cover-glass and to each other.

5. While the initial stages in the formation of the ectodermal membrane were accomplished by migration and flattening out upon the under surface of the mesenchyme of the cells originally covering the body, the further extension of the membrane involved the formation and growth of new cells and tension exerted by the mesenchyme upon the thick edge of the ectoderm.

6. Mitosis was observed in several mesenchyme cells but not in the ectoderm, although new cell boundaries appeared from time to time. Frequently ectoderm cells contained two nuclei or one irregularly lobed nucleus.

7. During all stages in the formation of the ectodermal membrane the movement of the cells is a mass movement. Their reactions are much slower than those of the mesenchyme, and are never amœboid in character.

8. There is an essential similarity in the outgrowth of the ectodermal membrane and the process of wound-healing in respect to (*a*) the mass migration of the ectoderm cells, and (*b*) the stereotropic activity of the cells which is evoked by contact with the mesenchyme.

I wish to acknowledge my indebtedness, for the valuable suggestions and criticism, to Prof. W. H. Lewis and Mrs. Lewis, of the Department of Embryology of the Carnegie Institution of Washington, under whose direction this work was accomplished.

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DESCRIPTION OF PLATES.

Figs. 1–10 are from photographs of cultures fixed in Zenker's solution without acid, and stained in iron hematoxylin. All figures except 1 and 5 were made with the high power. 1 and 5 were photographed with No. 4 Oc. and No. 16 lens.

PLATE I.

FIG. 1. Seven-day culture from 15-day embryo, showing extent of double membrane composed of ectoderm and mesenchyme. The latter projects beyond the thickened edge of the ectoderm in the form of a loose reticulum or of isolated cells.

FIG. 2. Portion of outgrowth from an explant showing a continuous ectodermal membrane of large flat cells, and an imperfect membrane of darker granular mesenchyme cells, which lies above it.

FIG. 3. Portion of a reticulum of elongated mesenchyme cells, attached at one end to the thickened edge of the ectoderm, anchored at the other to the cover-glass by broad fan-shaped expansions. In some of the ectoderm cells delicate striations are visible.

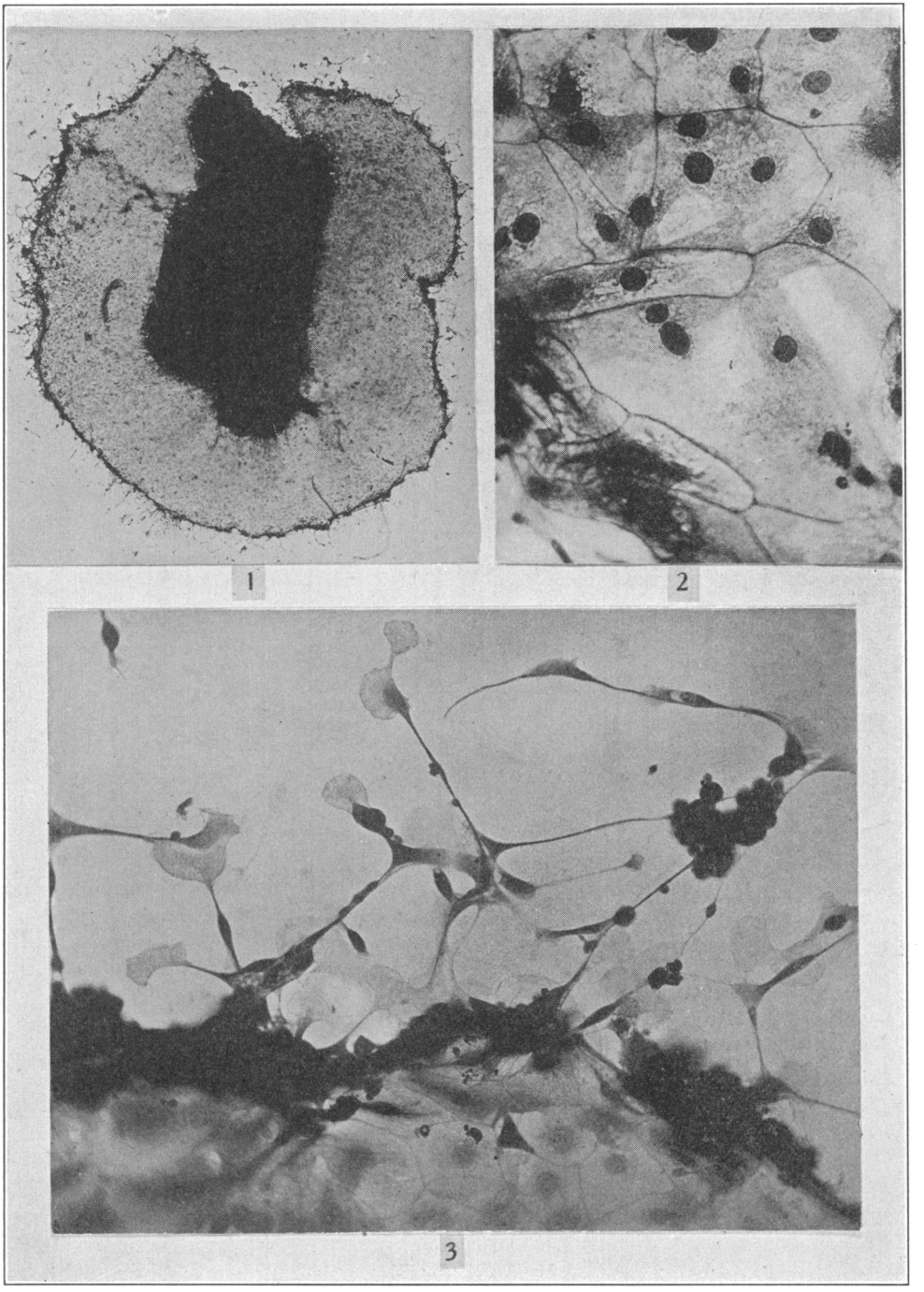

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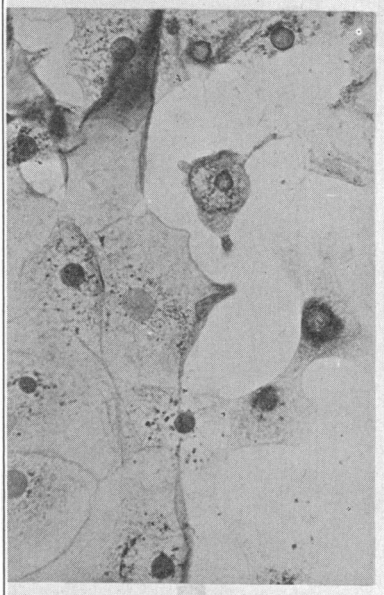
PLATE II.

FIG. 4. Portion of edge of ectodermal membrane, the cells almost completely flattened and slightly curled back at their extreme edge; mesenchyme cells projecting beyond them.

FIG. 5. Result of contraction of the ectoderm due to mechanical disturbance. The dark mass at the left is a retracted membrane of ectoderm and mesenchyme. Other portions of the mesenchyme remained adhering firmly to the cover-glass.

FIG. 6. Group of ectoderm cells, with large nuclei and faintly granular cytoplasm. Nuclei of mesenchyme cells are smaller, and are surrounded by dark granules derived from degenerated pigment cells; the boundaries of mesenchyme cells are not visible.

FIG. 7. Group of yolk cells lying upon the mesenchymal membrane where they had migrated from the digestive tract. The yolk spheres are stained deep black with hematoxylin.



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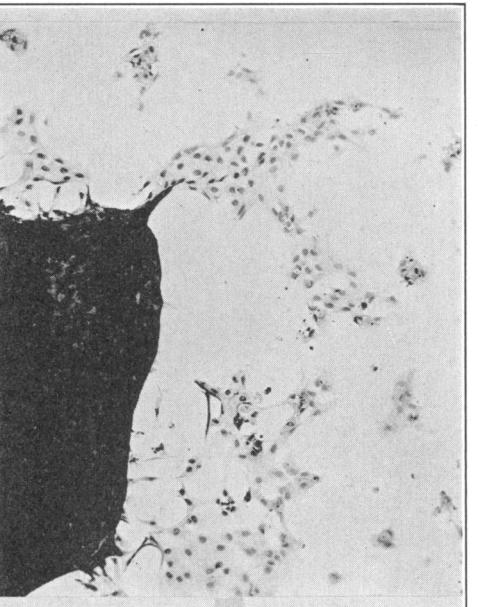

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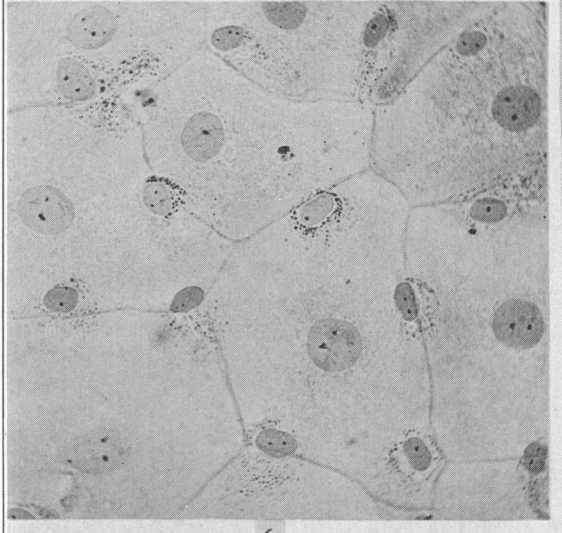

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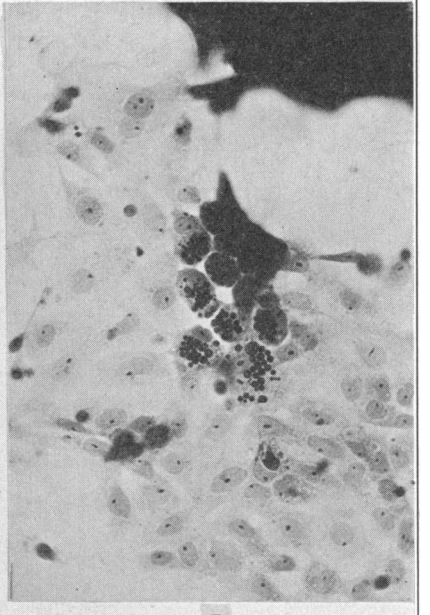

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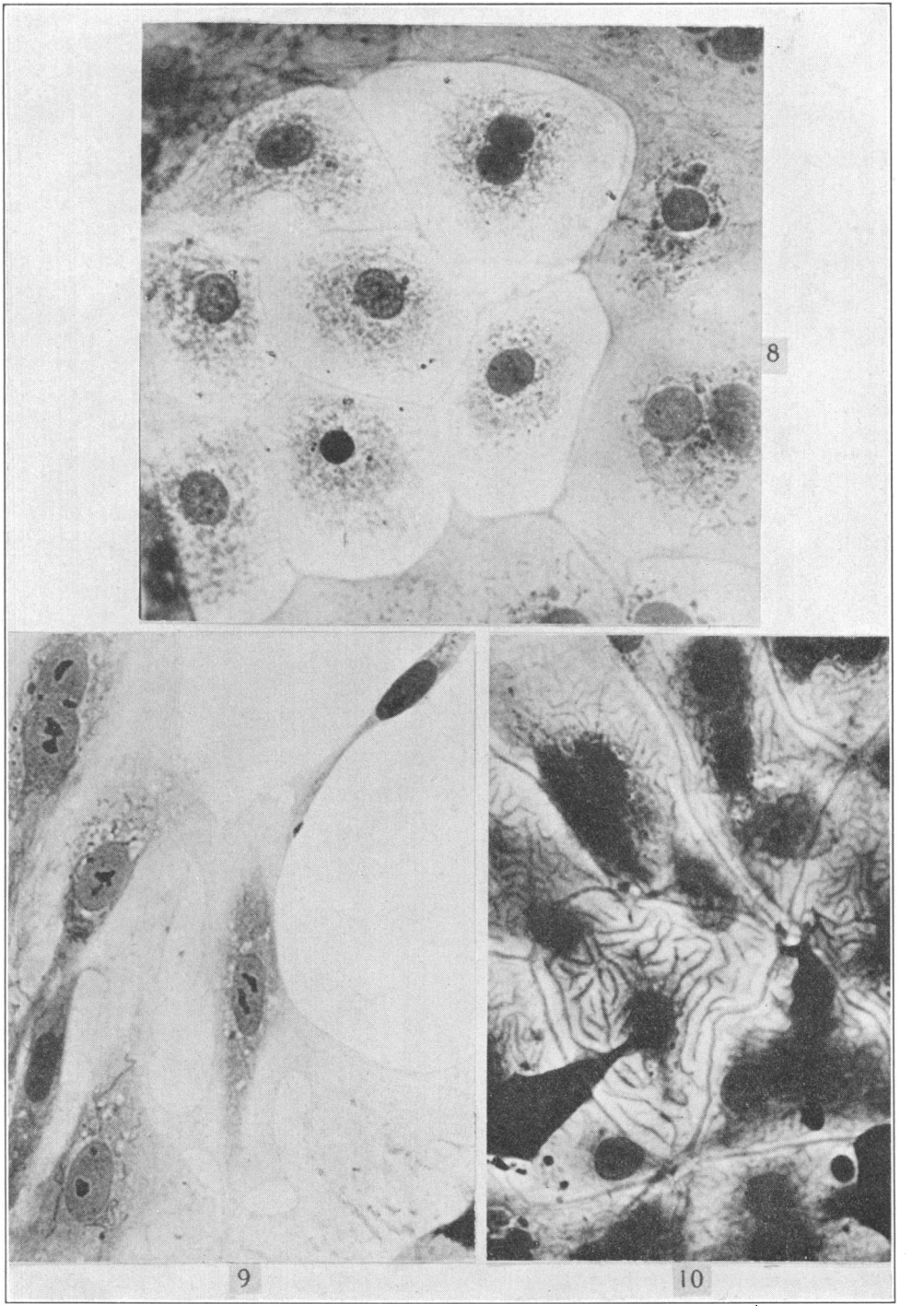
PLATE III.

FIG. 8. Group of seven ectoderm cells, free from overlying mesenchyme. A bilobed nucleus is visible in one cell.

FIG. 9. Group of mesenchyme cells forming a membrane with relatively small intercellular spaces. Mitochondria are visible in the form of threads and granules.

FIG. 10. Portion of outgrowth showing striations in ectoderm cells. Nuclei and cytoplasm of mesenchyme cells are visible as dark masses upon the ectoderm.



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